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Increased apoptosis of trophoblasts from pregnancies complicated by intrauterine growth restriction is associated with aberrant Fas-associated death domain (FADD) expression

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Summary

Intrauterine growth restriction (IUGR) is one of the most common pregnancy complications worldwide. However, the pathogenesis of IUGR is not completely clear. The authors conducted this study to investigate trophoblast apoptosis and the underlying molecular mechanisms in fetal membranes from pregnancies complicated by IUGR, in order to further understand the potential pathological processes involved in human IUGR. Fetal membranes were obtained from patients with pregnancies affected by IUGR (n = 10) and normal term pregnancies (n = 10). Nine of the ten delivered infants had a birth weight below the 10th percentile for gestational age, accompanied by morphological differences, and other extensive lesions common in IUGR cases. The fetal membranes were subjected to enzymatic digestion and flow cytometric analysis to evaluate apoptosis, as well as determination of cleaved caspase-3 and poly (ADP-ribose) polymerase (PARP) products. Expression of Fas-associated death domain (FADD) protein was detected using Western blot. Increased apoptosis was observed in the trophoblasts of fetal membranes from IUGR cases compared with normal control pregnancies. Trophoblasts from the fetal membranes of IUGR pregnancies exhibited consistently increased expression of the FADD protein compared to those from the normal pregnancies. The present results revealed significantly enhanced apoptosis and aberrant FADD expression in the trophoblasts from fetal membranes obtained from pregnancies complicated by IUGR compared with normal pregnancies. The disrupted balance of trophoblast apoptosis may be associated with aberrant FADD expression, which contributes to the pathological processes associated with IUGR.

Key words: IUGR; Apoptosis; FADD; Trophoblasts; Placenta.

Introduction

Intrauterine growth restriction (IUGR) is one of the most common pregnancy complications worldwide [1]. IUGR is associated with a higher risk of perinatal mortality, and permanent risk for hypertension, renal or cardiovascular disorders in the fetus and neonate [2]. Despite the varied causes of IUGR, including infections, vascular or placental disorders in pregnancy, the definite pathogenesis of IUGR remains unclear. Thus, more knowledge is required to improve therapeutic strategies for human IUGR.

As one of the programmed death pathways, apoptosis plays a crucial role in various physiological and pathological processes. During normal pregnancies, apoptosis is important for maintaining the normal structure and function of the placenta and embryo [3]. Apoptosis normally occurs in both the maternal and fetal tissues, and is involved in placental development, including the differentiation and turnover of villous trophoblasts, trophoblast invasion, and the transformation of spiral arteries [4]. Disruption of normal apoptosis processes is a basic pathological mechanism involved in human pathological states associated with pregnancy [5].

Fas-associated death domain (FADD) protein, also known as Mort-1, is a signal transducer of death receptor signaling. The death domain of the FADD protein interacts with death domain of Fas, resulting in the initiation of apoptosis [6]. FADD has been shown to play important roles in various physiological processes, including T cell immunity and sensitivity of cancer cells to drugs and cell motility [7, 8]. Previous studies have reported that FADD has multiple roles, particularly regulation of apoptosis, in the development of normal healthy embryos [9, 10]. Altered FADD expression has also been found to be associated with the pathogenesis of different diseases. However, it is still unclear whether dysregulation of FADD is associated with the progression of human IUGR.

In the present study, the authors aimed to investigate the incidence of apoptosis in fetal membranes from pregnancies complicated by IUGR compared with normal pregnancies. The authors further aimed to describe the novel role of FADD dysregulation in the regulation of trophoblast apoptosis, which is known to contribute to the progression of human IUGR.

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Table 1. — *Demographic characteristics of pregnancies.*

Characteristics	Control	IUGR
No.	10	10
Mean age of mother (years)	25 ± 5	26 ± 5
Gestation age (weeks)	35.5 ± 1.5	39 ± 0.99
Nulliparous	8	10
Multiparous	2	0
Vaginal delivery	4	3
Cesarean delivery	6	7
Male infants	4	3
Female infants	6	7

Table 2. — *Clinical characteristics of pregnancies.*

Samples		Gestation age (weeks)	Mode of delivery	Membrane status	Birth weight
IUGR	1	32	NSVD	SRM	<10%
	2	36	CSNIL	I-ARM	<10%
	3	37	CSNIL	I-ARM	<10%
	4	36	CSNIL	SRM	<10%
	5	36	CSNIL	I-ARM	<10%
	6	35	CSNIL	I-ARM	<10%
	7	36	NSVD	SRM	<10%
	8	37	CSNIL	SRM	<5%
	9	34	NSVD	SRM	<10%
	10	36	CSNIL	I-ARM	<10%
Control	1	39	NSVD	SRM	-
	2	39	NSVD	SRM	-
	3	40	CSNIL	I-ARM	-
	4	40	CSNIL	I-ARM	-
	5	40	CSNIL	I-ARM	-
	6	37	NSVD	SRM	-
	7	40	CSNIL	I-ARM	-
	8	38	CSNIL	I-ARM	-
	9	39	CSNIL	I-ARM	-
	10	39	NSVD	SRM	-

NSVD: normal spontaneous vaginal delivery. CSNIL: caesarean section, not in active labour. SRM: spontaneous rupture of membrane. I-ARM: artificial rupture of intact membrane.

Materials and Methods

This study was approved by the Human Studies Committee of Xi'an No. 4 Hospital, and informed consent was obtained from all patients. The work was done following the standards set by the Declaration of Helsinki (2013).

Fetal membranes were obtained from patients affected by IUGR pregnancies (n = 10) and normal term pregnancies (n = 10). The diagnosis and inclusion criteria for IUGR was defined as a fetal weight below the 10th percentile for gestational age. Patients with a history of smoking or drug abuse, infection, pre-eclampsia or other complications during pregnancy were excluded from the study. The IUGR and normal pregnancies were matched for gestational age, and randomly selected. Tables 1 and 2 depict the clinical characteristics of all subjects that participated in the study. The fetal membranes collected were subjected to enzymatic digestion prior to performing an apoptosis assay and protein expression analysis.

Cell apoptosis assay was performed using a detection kit. Briefly, after the enzymatic digestion of fetal membranes, trophoblast cells (5×10^6 cells/well) were harvested with ice-cold PBS and resuspended with binding buffer, followed by staining with annexin V-fluorescein isothiocyanate and propidium iodide (PI). The stained cells were then analyzed by flow cytometry.

The total proteins isolated from the fetal membrane cells were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes. The blots were incubated with primary antibodies (rabbit anti-cleaved caspase-3 and anti-PARP, 1:1000, and mouse anti-FADD, 1:1000; mouse anti-β-actin, 1:3000) at 4ºC overnight, then detected with secondary antibodies for one hour at room temperature. Bands were analyzed by enhanced chemiluminescence (ECL).

Data for the apoptosis and protein expression analyses are expressed as mean ± SEM. GraphPad Prism 6 was used to perform all statistical analyses. The differences between groups were analyzed using Student's *t*-test. A Bonferroni correction was used to adjust for multiple testing. A p-value < 0.05 was considered statistically significant.

Results

The group of pregnancies (n=10) met the inclusion criteria for IUGR in the present study. For use as controls, the authors identified and randomly selected normal pregnancies (n=10) that were matched for gestational age with those pregnancies complicated by IUGR. Table 1 shows the primary and clinical characteristics of the selected IUGR cases, as well as the controls. The decreased birth weight of delivered infants was the main indication of IUGR cases compared to their matched controls. Nine of the ten delivered infants had a birth weight below the 10th percentile for their gestational age (Tables 1 and 2). The placentas from IUGR cases were also morphologically different from the controls. The authors also noted other symptoms in the IUGR group when compared to the matched controls, including accelerated maturation of villi, massive villous fibrin deposits, decidual vasculopathy, among others (data not shown). These findings were consistent with the extensive lesions often observed in both the maternal and fetal compartments for pregnancies complicated by IUGR.

The authors analyzed apoptosis in fetal membranes, specifically the trophoblast cells, in the IUGR group and control cases using two approaches. The authors firstly measured the apoptosis of trophoblast cells using flow cytometric analysis. As shown in Figure 1A, trophoblast cells from fetal membranes of IUGR cases exhibited consistently higher levels of apoptosis compared with those collected from normal control pregnancies. They observed consistent results for the quantitative analysis of apoptosis (Figure 1B). The authors also demonstrated abnormal apoptosis of trophoblasts at the molecular level using Western blotting. The results indicated that the levels of the apoptosis factors cleaved caspase-3 and poly-ADP-ribose polymerase (PARP, 85 kDa) were significantly enhanced in the trophoblast cells of fetal membranes from IUGR cases,

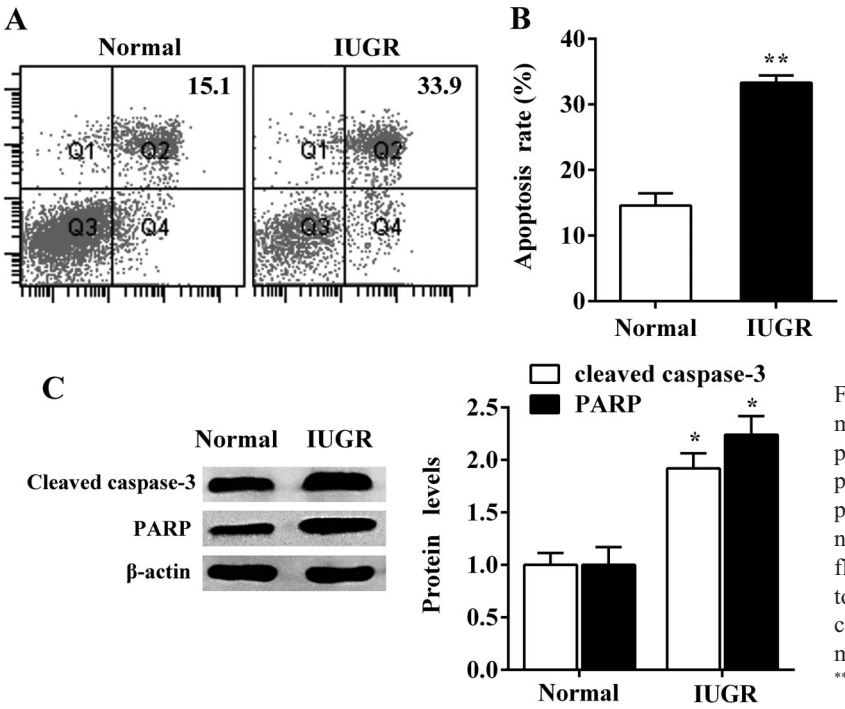

Figure 1. — Apoptosis of trophoblasts in fetal membranes from human IUGR and normal pregnancies. (A) Representative figure of trophoblasts apoptosis in fetal membranes from pregnancies complicated by IUGR and from normal control pregnancies was examined using flow cytometric analysis. (B) Quantified apoptosis rate. (C) Protein expression levels of cleaved caspase-3 and poly-ADP-ribose polymerase (PARP), as detected by Western blotting. $^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* control.

whereas low levels of cleaved caspase-3 and PARP were observed in trophoblasts from normal control pregnancies (Figure 1C). Taken together, these results reveal that the placentas, especially the fetal membranes, from pregnancies complicated by IUGR exhibited an enhanced apoptosis rate, which may contribute to the progression of IUGR.

To identify the potential mechanisms involved in the regulation of apoptosis in placentas during pregnancy, the authors analyzed the expression of FADD in the fetal membranes collected from pregnancies complicated by IUGR and from normal control pregnancies. Expression of the FADD protein was measured using Western blotting with an anti-FADD antibody, and the housekeeping gene β-actin was used as the internal reference. Representative blots are shown in Figure 2A, in which a 45 kDa protein (FADD) was detected in the fetal membrane tissues from pregnancies complicated by IUGR. However, this specific band was only weakly detectable in the fetal membranes from normal control cases. Immunoblotting with an anti-β-actin antibody exhibited a 42 kD band (β-actin) in the fetal membranes from both IUGR and normal control pregnancies.

The immunoblotting of the FADD protein was then normalized for the expression of the housekeeping β-actin protein, then semi-quantitatively analyzed. As shown in Figure 2B, there was a three-fold increase in the expression of FADD in fetal membranes from pregnancies complicated by IUGR compared to those from normal control pregnancies. These findings suggest that dysregulation of the FADD protein may play a role in disrupting the apoptosis of cells in placental membranes during pregnancies complicated by IUGR.

Discussion

In the present study, the authors investigated the apoptosis of cells in fetal membranes from pregnancies complicated by IUGR, as well as from normal pregnancies, in order to increase their understanding of the potential pathological processes involved in human IUGR. The cases of IUGR pregnancies included in the present study met the diagnosis criteria of IUGR, defined as the low birth weight of delivered infants, in addition to displaying other characteristic features, including comprehensive lesions in both the maternal and fetal compartments.

Trophoblasts, including syncytiotrophoblasts and cytotrophoblasts, are important components of fetal membranes and the associated villi, which play an important role in the normal development of the placenta and nutrient transport between maternal and fetal compartments [5]. The balance of proliferation, differentiation, and apoptosis of trophoblasts is crucial for the normal development of fetal membranes during pregnancy [11-13]. The disruption of trophoblast apoptosis has been identified as one of the major pathologies related to various pregnancy complications, including fetal growth restriction (FGR) and IUGR [2, 14, 15].

In this study, the authors assessed the apoptosis of fetal membranes collected from pregnancies complicated by IUGR, as well as normal cases, using two approaches.

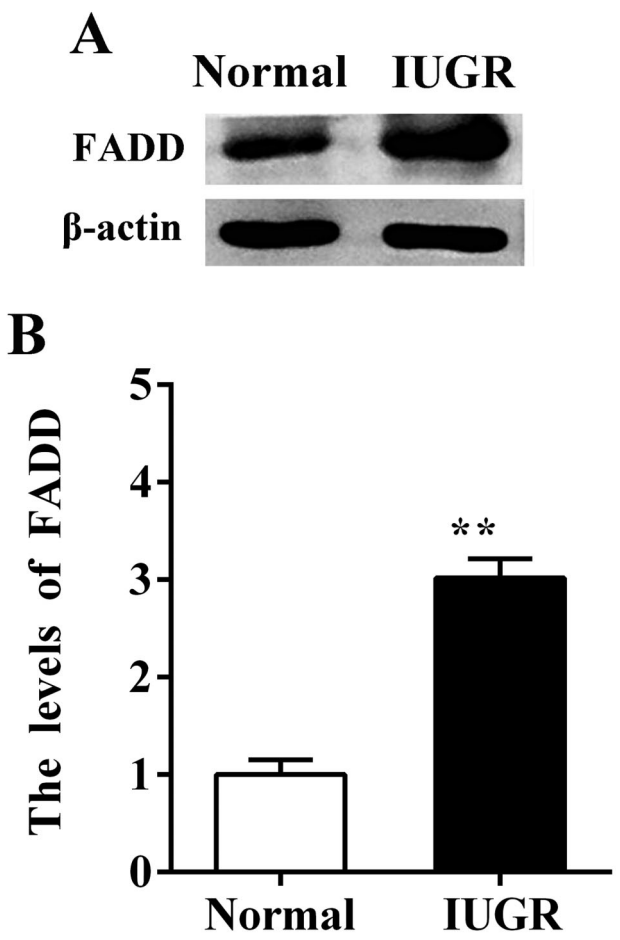

Figure 2. — Aberrant FADD expression in trophoblasts from fetal membranes of IUGR pregnancies. (A) The expression of FADD in fetal membranes from pregnancies complicated by IUGR and normal control pregnancies was evaluated by Western blotting. (B) Quantified levels of the FADD protein. $^{**}p < 0.01$, $^{*}p < 0.05$ *vs.* control.

Firstly, the authors tested the level of apoptosis using flow cytometric analysis. The trophoblasts derived from fetal membranes from IUGR cases exhibited consistently increased apoptosis compared with those from normal control pregnancies. The authors then obtained supporting evidence for the finding of increased apoptosis by evaluating the protein expression of caspase-3 and PARP (85 kDa), two characteristic apoptosis cleavage products, via immunoblotting. These findings confirm the enhanced apoptosis in placentas, specifically the trophoblasts of fetal membranes and villi, from pregnancies complicated by IUGR.

Another approach used in the current study to clarify the pathogenesis of IUGR was to investigate the potential mechanisms involved in the regulation of apoptosis of fetal membranes. The authors found that FADD, the regulator of apoptosis, was highly expressed in fetal membrane tissues from pregnancies complicated by IUGR compared with the normal cases. FADD is a signal transducer of death receptor signaling, and plays a crucial role in the induction of cell apoptosis by interacting with the death signaling of Fas [6]. Various studies have reported that FADD has multiple functions during embryogenesis, and is essential for the development of normal embryos [9, 10, 16]. By interacting with caspase or NF-κB-related signaling, FADD has been associated with the regulation of T cell immunity, sensitivity of cancer cells to drugs, as well as cell proliferation and death [7, 8]. Therefore, the authors speculate that some negative factors could result in enhanced expression of the FADD protein. Trophoblast apoptosis may be induced by an increase in the aberrantly-expressed FADD protein, which could disrupt the turnover of trophoblasts, contributing to the impaired function of fetal membranes, and consequently, resulting in IUGR.

Although the present findings of increased trophoblast apoptosis and enhanced FADD expression associated with pregnancies complicated by IUGR was novel, this study is not without limitations. The majority of cells isolated from fetal membranes appeared to be trophoblast cells, however, it was difficult to distinguish these from decidual cells due to limitations associated with the analysis methods. In addition, the effect of FADD on apoptosis is complex, as various different proteins and/or factors may be involved in the regulation of FADD-related pathways, such as Fas, caspase-3, and Bcl-3 [7, 8]. Hence, evaluation of underlying interactions and further clarification of the mechanisms are important future directions for this research, in order to further elucidate the pathological processes of IUGR.

In conclusion, the present results reveal significantly enhanced apoptosis and aberrant FADD expression of trophoblasts obtained from the fetal membranes of pregnancies complicated by IUGR when compared with normal pregnancies. The disrupted balance of trophoblast apoptosis may be associated with the aberrant expression of FADD, which may contribute to the pathological processes associated with IUGR.

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